ERYTHROVIRUS AND ITS APPLICATIONS

The present invention relates to nucleic sequences derived from a human erythrovirus, to their fragments as well as to their applications as diagnostic reagent and as an immunogenic agent.

Sero-epidemiological studies show that infection with the parvovirus B19, recently renamed erythrovirus B19, is commonly and widely distributed worldwide.

In Europe, the seroprevalence for erythrovirus B19 is about 10% in subjects under 5 years, about 50% for subjects over 20 years and greater than 90% in elderly persons.

The high seroprevalence rate suggests that erythrovirus B19 is highly contagious. During epidemics, the rate of transmission to subjects in close contact is 10 to 60%, the route of transmission being mainly aerial (respiratory secretions).

Erythrovirus B19 is a specifically human virus. Acute infection commonly causes benign maculopapular skin rashes in children (epidermal megalerythema or 5th disease). Arthralgias may accompany the rashes and may exceptionally become chronic.

A transient acute erythroblastic attack usually occurs in patients already carrying a chronic haemolytic anaemia (sickle cell anaemia, thalassaemia, pyruvate kinase deficiency and the like), causing a transient aregenerative acute anaemia.

Acute primary infection with erythrovirus B19 is particularly dangerous in pregnant women with a risk of transmission to the foetus estimated at 30%. The risk of foetal death by anaemia, hepatic insufficiency, cardiac insufficiency and foetoplacental anasarca is estimated at between 5 and 9%.

Chronic infections with erythrovirus B19 are found essentially in immunosuppressed subjects (chronic myeloid leukaemia, humoral and cellular immune

deficiency, organ or marrow transplants, AIDS diseases).

In seropositive HIV-1 patients, chronic infection with erythrovirus B19 is responsible for chronic anaemia, but can also act on the other lineages (neutropenia and especially thrombopenia). The absence of a sufficient humoral immune response in these patients allows the installation of a chronic erythroviraemia and explains both the chronic erythroblastopenia and the absence of other symptoms such as rash or arthralgias.

Erythrovirus B19 is a virus having a single-stranded DNA genome of about 5.4 kbases; it is the only erythrovirus classified to date; all the strains which have been sequenced and which have been the subject of a publication in the sequence libraries (GenBank or EMBL) exhibit a low genetic variability (98% nucleic sequence similarity over the whole genome and 96% similarity over the VP1 region) (R.O. SHADE, *J. Virol.*, 1986, 58, 3, 921-936, B19-AU).

Virological diagnosis of erythrovirus B19 infections is based essentially on the detection of the viral genome, insofar as the culture cannot be carried out routinely.

For acute infections with erythrovirus B19 (primary infections), this detection can be made by gene amplification (PCR), but also be hybridization (*dot-blot*) given the viral titre, which is usually very high during primary infections (up to 10^{14}/ml of serum); however, the viral titre is much lower during chronic infections and only a gene amplification detection method can be envisaged.

These detection techniques are dependent on the genetic variability of the virus tested for; the reagents prepared from known erythrovirus B19 sequences do not make it possible to detect the variant erythrovirus infections, either by gene amplification or by B19 serodiagnosis.

Indeed, the existing serodiagnostic tests are specific for erythrovirus B19 (International Application PCT WO 91/12269; International Application PCT WO 96/09391 (IDEIA® Parvovirus B19 IgG and IgM, DAKO; Parvovirus B19 IgG and IgM *Enzyme Immunoassay*, BIOTRIN)).

Consequently, the detection techniques specified above risk producing negative results both at the nucleic level and with respect to the antibody response.

The identification and the taking into account of new variants are important for developing:

- reagents for the detection and diagnosis of human erythrovirus infections (serodiagnosis, PCR, hybridization), which are sufficiently sensitive and specific, that is to say which do not lead to false-negative or false-positive results,

- compositions capable of protecting against all erythrovirus infections (vaccines), and

- compositions capable of treating a variant erythrovirus infection (serotherapy, monoclonal antibodies).

The inventors therefore set themselves the aim of providing erythrovirus-derived sequences capable of allowing the detection of a variant erythrovirus (called erythrovirus type V9), that is to say which is genetically distant from erythrovirus B19.

The subject of the present invention is a nucleic acid sequence, characterized in that it is selected from the group consisting of:

- the sequences derived from an erythrovirus which, molecularly, cannot be recognized as an erythrovirus B19 because it exhibits a genetic divergence or distance $\geq$ 10% ($<$ 90% similarity) over the whole genome with respect to the erythrovirus B19 sequences and which exhibit a genetic divergence of less than or equal to 6% ($>$ 94% similarity) with respect to the sequence SEQ ID NO:1,

- the sequence SEQ ID NO:1, and

- the nucleotide sequences capable of hybridizing under stringent conditions with the said sequence ID NO:1.

This variant erythrovirus is called type V9 variant.

Stringent conditions are understood to mean, for the purposes of the present invention, the following conditions:

. hybridization for 3 to 24 h in a 1XSSC buffer containing 50% formamide, at 42°C, and

. 3 washes of 15 min in a 2XSSC buffer, at 60°C.

The sequence SEQ ID NO:1, which corresponds to about 95% of the genome of an erythrovirus type V9 and which includes all the coding sequences, has a restriction map which is different from that of the B19 erythroviruses, in particular as regards the BamHI site (no site), HINDIII site (only one site) and PvuII site (five sites).

More precisely, the sequence SEQ ID NO:1 has a restriction profile which is different from that of erythrovirus B19, in particular by the following restriction sites: AccI, AflIII, AlwI, AlwNI, **ApaI**, AvaI, AvaII, AvrII, **BamHI**, BanI, BanII, BbeI, BbsI, BceFI, BcgI, BcnI, **BglII**, BsgI, BsiEI, BsmI, BsmAI, Bsp120I, BspHI, BspMI, BsrFI, Bst1107I, BstEII, BstUI, Bsu36I, DpnI, DraIII, DsaI, EaeI, EagI, EarI, Ec1136I, EcoNI, Eco109I, EcoRI, EheI, FokI, HaeI, HaeIII, HgaI, HgiAI, HhaI, HincII, **HindIII**, HinPI, HpaI, KasI, MaeII, MboI, McrI, MscI, **MunI**, NarI, NciI, NcoI, NsiI, NspI, Nsp7524I, NspBII, NspCI, PflMI, PmeI, Ppu10I, PpuMI, PstI, **PvuII**, SacI, Sau3AI, ScaI, SfaNI, SfcI, SmaI, SpeI, SphI, SspI, StuI, StyI, SwaI, Tth111I, XbaI, XmaI and their isoschizomers.

The subject of the present invention is also fragments of sequence ID NO:1 which are capable of allowing the detection of an erythrovirus V9 and characterized in that they comprise a nucleotide sequence selected from the group consisting of:

a) a sequence corresponding to positions 328-2340 of SEQ ID NO:1, encoding the NS1 protein (SEQ ID NO:81),

b) a sequence corresponding to positions 1796-2017 of SEQ ID NO:1, encoding the 7.5 kDa protein (SEQ ID NO:83),

c) a sequence corresponding to positions 2336-4678 of SEQ ID NO:1, encoding the VP1 protein (SEQ ID NO:85),

d) a sequence corresponding to positions 2336-3016 of SEQ ID NO:1, encoding the VP1u (SEQ ID NO:87),

e) a sequence corresponding to positions 2523-2828 of SEQ ID NO:1, encoding the X protein (SEQ ID NO:89),

f) a sequence corresponding to positions 3017-4678 of SEQ ID NO:1, encoding the VP2 (SEQ ID NO:91),

g) a sequence corresponding to positions 4488-4883 of SEQ ID NO:1, encoding the 11 kDa protein (SEQ ID:93),

h) a nucleotide sequence capable of hybridizing with one of the sequences SEQ ID NO:1, SEQ ID NO:81, SEQ ID NO:83, SEQ ID NO:85, SEQ ID NO:87, SEQ ID NO:89, SEQ ID NO:91 or SEQ ID NO:93,

i) the sequences SEQ ID NO:2-80,

j) the sequences SEQ ID NO:105 (E1905f), 106 (E1987r), 107 (E2076f), 108 (E2151r), 109 (E2406r), 110 (E2149rs), 111 (E2717f), 112 (E2901r), 113 (e1855f), 114 (e2960r), 115 (e1863f), 116 (e2953), 117 (e2435fStul/BglII), 118 (e4813rEcoRI), 119 (e3115fBamHI), 120 (e4813rBamHI) and 121 (e1954fp) and

k) the sequences complementary to the preceding sequences, the fragments derived from the preceding sequences of at least 17 nucleotides or their complementary sequences.

For the purposes of the present invention, nucleic sequence or nucleotide sequence (DNA or RNA sequence) is understood to mean one of the sequences as defined above and their complementary sequences (anti-sense sequences) as well as the sequences comprising one or more of the said sequences or fragments thereof.

The invention also includes nucleotide fragments complementary to the preceding ones as well as fragments modified with respect to the preceding

ones by removal or addition of nucleotides in a proportion of about 15%, with respect to the length of the above fragments and/or modified at the level of the nature of the nucleotides, as long as the modified nucleotide fragments retain a capacity for hybridization with the erythrovirus V9 DNA or RNA sequence which is similar to that exhibited by the corresponding unmodified fragments.

Some of these fragments are specific and are used as a probe or primer; they hybridize specifically to an erythrovirus V9 or a related erythrovirus; a virus related to erythrovirus V9 is understood to mean an erythrovirus exhibiting a genetic divergence of less than or equal to 6%; these fragments are selected from the group consisting of the sequences SEQ ID NO:45-80 and NO:108 and 110, or their complementary sequences, the sequences derived from these sequences of at least 17 nucleotides and the sequences comprising the said sequences and they find application in the specific identification of an erythrovirus V9 or of a related erythrovirus.

Others of these fragments are used as primers, for the amplification of sequences derived from an erythrovirus type V9 or a related virus, such as the sequence SEQ ID NO:1; these primers are chosen from the group consisting of the sequences SEQ ID NO:2-44 and the sequences SEQ ID NO:105-109 and 111-121 or their complementary sequences and the sequences derived from the said sequences, of at least 17 nucleotides.

The said fragments also include, in the case of primers, the antisense sequences.

Such sequences find application for the differential identification of erythroviruses (erythrovirus B19 and erythrovirus V9), combined with probes as defined above and/or with suitable restriction enzymes.

The said primers preferably comprise between 17 and 30 nucleotides; preferred primers are the following: the sequence SEQ ID NO:105 (positions 1797-

1815 of the sequence SEQ ID NO:1), which corresponds to the sequence SEQ ID NO:10, the sequence SEQ ID NO:106 (positions 1899-1879 of the sequence SEQ ID NO:1), which corresponds to a fragment of the antisense sequence of the sequence SEQ ID NO:11, the sequence SEQ ID NO:107 (positions 1968-1987 of the sequence SEQ ID NO:1), which corresponds to a fragment of the sequence SEQ ID NO:13, the sequence SEQ ID NO:108 (positions 2061-2043 of the sequence SEQ ID NO:1), which corresponds to a fragment of the antisense sequence of the sequence SEQ ID NO:58, the sequence SEQ ID NO:109 (positions 2317-2298 of the sequence SEQ ID NO:1), which corresponds to a fragment of the antisense sequence of the sequence SEQ ID NO:16, the sequence SEQ ID NO:111 (positions 2609-2627 of the sequence SEQ ID NO:1), which corresponds to a fragment of the sequence SEQ ID NO:19 and the sequence SEQ ID NO:112 (positions 2812-2793 of the sequence SEQ ID NO:1), which corresponds to a fragment of the antisense sequence of the sequence SEQ ID NO:23.

Preferred pairs of primers are the following:

- pair A: primers SEQ ID NO:111 and SEQ ID NO:112;
- pair B: primers SEQ ID NO:105 and SEQ ID NO:106;
- pair C: one of the sequences SEQ ID NO:2-44, 105, 106, 107, 109, 111 or 112 and one of the sequences SEQ ID NO:45-80, 108 or 110;
- pair D: primer SEQ ID NO:107 and primer SEQ ID NO:109;
- pair E: two primers selected from the sequences SEQ ID NO:2-44, 105, 106, 107, 109, 111 or 112;
- pair F: two primers selected from the sequences SEQ ID NO:45-80, 108 or 110.

These various primers can be used, depending on the fragment amplified, as sense primer or as antisense primer.

The subject of the present invention is also a variant erythrovirus, characterized in that its genome cannot be recognized molecularly as an erythrovirus B19, in that it exhibits a divergence of less than or equal to 6% with the sequence SEQ ID NO:1, as defined above, and in that its genome hybridizes specifically, under stringent conditions, as defined above, with one of the sequences SEQ ID NO:45 to 80, 108 and 110, as defined above.

The subject of the present invention is also a plasmid, characterized in that it comprises the viral genome of a variant erythrovirus strain, called erythrovirus V9 or a fragment thereof, which cannot be recognized molecularly as an erythrovirus B19 and which exhibits with the latter a genetic divergence of $\geq 10\%$ over the whole genome with respect to the erythrovirus B19 sequences and a divergence of less than or equal to 6% with the sequence SEQ ID NO:1.

The viral genome of the said erythrovirus V9 is considered to be genetically distant from erythrovirus B19.

According to an advantageous embodiment of the said plasmid, it includes the sequence SEQ ID NO:1 (PCD.V9.C22).

The subject of the present invention is also a diagnostic reagent for the differential detection of type V9 erythroviruses, characterized in that it is selected from the sequences SEQ ID NO:45-80, 108 and 110, optionally labelled with an appropriate marker.

Among the appropriate markers, there may be mentioned radioactive isotopes, enzymes, fluorochromes, chemical markers (biotin and the like), haptens (digoxygenin and the like) and antibodies or appropriate base analogues.

The subject of the present invention is also a process for the rapid and differential detection of erythroviruses, by hybridization and/or gene amplification, using a biological sample as starting

material, which process is characterized in that it comprises:

(1) a step in which a biological sample to be analysed is brought into contact with at least one probe of sequence SEQ ID NO:45-80, 108 or 110, and

(2) a step in which the product(s) resulting from the erythrovirus nucleotide sequence-probe interaction is (are) detected by any appropriate means.

Preferably, the hybridization comprises a pre-hybridization which is carried out in a buffer which comprises 5-60% of formamide; 1-5X SSC; 2% of blocking reagent (*Blocking buffer*, Boehringer Mannheim, Meylan, France); 0.1% of N-laurylsarcosine; 0.01-5% of SDS, at 40-70°C for 90 minutes, and then the hybridization is carried out in 3 ml of a buffer of the same composition with 10 µl of labelled probe at 40-70°C for 1-30 hours.

In accordance with the said process, it may comprise, prior to step (1):

. a step extracting the nucleic acid to be detected, belonging to the virus genome, which may be present in the biological sample, and

. at least one gene amplification cycle.

The gene amplification step is in particular carried out with the aid of one of the following gene amplification techniques: amplification with Qβ-replicase (I. Haruna et al., Proc. Nat. Acad. Sci. USA, 1965, 54, 579-587), PCR (polymerase chain reaction) (R.K. Saiki et al., 1986, Nature, 324:163-6), LCR (*ligase chain reaction*) (F. Barany, Proc. Nat. Acad. Sci. USA, 1991, 88, 189-193), ERA (*end-run amplification*) (C. Adams et al., 1994, *Novel amplification technologies for DNA/RNA-based diagnostics meeting*, San Francisco, CA, United States), CPR (*cycling probe reaction*) (P. Duck et al., Bio-techniques, 1990, 9, 142-147) or SDA (*strand displacement amplification*) (GT. Walker, 1994, *SDA: novel amplification technologies for DNA/RNA-based diagnostics meeting*, San Francisco, CA, United States).

According to an advantageous embodiment of the said process, the amplification cycles are carried out with the aid of a pair of primers selected from the sequences SEQ ID NO:2-44, 105-109 and 111-112 and fragments of these sequences, preferably from the pairs of primers as defined above.

When pair A is used, the amplification product is advantageously screened by the action of the restriction enzyme ApaI (GGGCCC): the product of amplification of a B19 genome is cleaved with ApaI (generating 2 fragments of 149 and 55 base pairs (bp)) whereas the product of amplification of a V9 genome is not cleaved by ApaI (a fragment of 204 bp); an agarose or acrylamide gel electrophoresis makes it possible to distinguish between these restriction fragments.

When pair B is used, the product of amplification is advantageously screened by the action of one of the following restriction enzymes: BglII (AGATCT), or MunI (CAATTG); different fragments are thus obtained depending on whether an erythrovirus V9 or B19 is involved; a fragment which comprises a BglII restriction site is specific for the variant erythrovirus V9 as defined above, whereas the B19 erythroviruses comprise an MunI site in this region. The product of amplification of a B19 genome is cleaved with MunI (generating 2 fragments of 36 and 67 bp) and is not cleaved by BglII (a fragment of 103 bp) whereas the product of amplification of a V9 genome is cleaved by BglII (2 fragments of 19 and 84 bp) and is not cleaved by MunI (a fragment of 103 bp); an agarose or acrylamide gel electrophoresis makes it possible to distinguish between these different restriction fragments.

When pair C is used (a primer capable of hybridizing with all erythroviruses and a primer capable of specifically hybridizing with erythrovirus V9) or when pair F is used (two primers capable of specifically hybridizing with erythrovirus V9), the V9

genome is amplified whereas there is no specific amplification with the B19 genome.

When pair D is used, the product of amplification is advantageously screened by hybridization with a labelled specific probe for erythrovirus V9, selected from the sequences SEQ ID NO:58-60 and 110, preferably by hybridization with the probe of sequence SEQ ID NO:110; the product of amplification of a V9 genome hybridizes specifically with these probes and in particular the probe of sequence SEQ ID NO:110, whereas the product of amplification of a B19 genome does not hybridize with the abovementioned probes.

When pair E is used, the product of amplification is screened by any method of hybridization with a probe specific for erythrovirus V9, selected from the sequences SEQ ID NO:45-80, 108 and 110; in this case, the product of amplification of a V9 genome hybridizes with the probe, but not the product of amplification of a B19 genome.

The subject of the invention is also the use of the sequences described above, of fragments derived from these sequences or of their complementary sequences, for carrying out a method of hybridization or of gene amplification of erythrovirus nucleic sequences, these methods being applicable to the *in vitro* diagnosis of the potential infection of an individual with an erythrovirus type V9.

The subject of the present invention is also a method of screening and typing an erythrovirus V9 or a related virus, characterized in that it comprises bringing a probe selected from the group consisting of the sequences SEQ ID NO:45-80, 108 and 110, optionally labelled, into contact with the nucleic acid of the virus to be typed and detecting the nucleic acid-probe hybrid obtained.

The subject of the present invention is also products of translation, characterized in that they are encoded by a nucleotide sequence as defined above.

The subject of the present invention is also a protein, characterized in that it is in particular capable of being expressed with the aid of a nucleotide sequence selected from the group consisting of the sequences SEQ ID NO:81, 83, 85, 87, 89, 91 and 93, as defined above and the derived peptides comprising between 7 and 50 amino acids.

Peptide is understood to mean below both the proteins and the peptides, as defined above.

Such peptides are in particular capable of being recognized by antibodies induced by an erythrovirus V9 and/or of inducing the production of anti-erythrovirus V9 antibodies.

The said peptides are in particular selected from the sequences SEQ ID NO:82 (NS1 protein), SEQ ID NO:86 (VP1 protein), SEQ ID NO:88 (single VP1 protein), SEQ ID NO:92 (VP2 protein) and SEQ ID NO:95-104, namely fragments of the VP1 protein [VP1a peptide (SEQ ID NO:95); VP1b peptide (SEQ ID NO:96); VP1c peptide (SEQ ID NO:97); peptide VP1d (SEQ ID NO:98); peptide VP1e (SEQ ID NO:99) and peptide VP1f (SEQ ID NO:100)], or fragments of the VP2 protein [peptide VP2a (SEQ ID NO:101); peptide VP2b (SEQ ID NO:102); peptide VP2c (SEQ ID NO:103); peptide VP2d (SEQ ID NO:104)] as well as the derived peptides comprising 7 to 50 amino acids.

The subject of the invention is also immunogenic compositions comprising one or more products of translation of the nucleotide sequences according to the invention and/or one or the peptides as defined above, obtained in particular by synthetic means.

The subject of the invention is also the antibodies directed against one or more of the peptides described above and their use for carrying out in particular a differential in vitro method of diagnosis of the infection of an individual with an erythrovirus.

The subject of the present invention is also a method for the immunological detection of an erythrovirus V9 infection, characterized in that it comprises:

- for the detection of anti-erythrovirus V9 antibodies, bringing a biological sample into contact with a peptide according to the invention (serodiagnosis),

- for the detection of erythrovirus V9 viral proteins, bringing a biological sample into contact with an antibody according to the invention;

the reading of the result being revealed by an appropriate means, in particular EIA, ELISA, RIA, fluorescence.

By way of illustration, such an in vitro method of diagnosis according to the invention comprises bringing a biological sample, collected from a patient, into contact with antibodies according to the invention or peptides according to the invention, and detecting, with the aid of any appropriate method, in particular with the aid of labelled anti-immunoglobulins, immunological complexes formed between the antigens or the antibodies of the erythroviruses which may be present in the biological sample and the said antibodies or the said peptides, respectively.

The reagents according to the invention are in particular useful for the detection of the V9 erythroviruses and related viruses in pregnant women, in HIV-positive patients with anaemia and/or chronic thrombopenia, recipients of organ or marrow transplants, and patients having a central acute anaemia and for whom the tests for the detection of erythrovirus B19 are negative.

The subject of the invention is, in addition, an erythrovirus diagnostic kit, characterized in that it includes at least one reagent according to the invention (probes, pairs of primers, peptides or antibodies).

In addition to the preceding features, the invention further comprises other features which will emerge from the description which follows, which refers to exemplary embodiments of the method which is the

subject of the present invention as well as to the appended drawings, in which:

- Figures 1, 2 and 3 illustrate phylogenetic trees for erythrovirus V9: Figure 1: phylogenetic tree for the complete erythrovirus sequence; Figure 2: phylogenetic tree for the erythrovirus NS1 genes; Figure 3: phylogenetic tree for the erythrovirus VP1 genes;

- Figures 4, 5 and 6 represent the genetic distances for the complete erythrovirus sequences (Figure 4), for the erythrovirus NS1 genes (Figure 5) and for the erythrovirus VP1 genes (Figure 6);

- Figure 7 illustrates the restriction map of sequence ID NO:1.

It should be understood, however, that these examples are given solely by way of illustration of the subject of the invention and do not constitute in any manner a limitation thereto.

EXAMPLE 1: Production of sequences conforming to the invention

An AatII/AatII restriction fragment of 5028 bp, representing virtually the entire (95%) genome of the V9 variant, was cloned into the sequencing vector pcDNA2.1 (Invitrogen, Netherlands) in the following manner.

The single-stranded viral DNA was extracted from the serum of a patient with an acute erythroblastopenic attack with the aid of a QIAamp Blood Kit column (Qiagen S.A., France). Using a step of hybridization in a 50 mM NaCl buffer at 56°C for 16 hours, the viral DNA is converted to double-stranded DNA. Next, 1.3 µg of double-stranded viral DNA is subjected to the AatII restriction enzyme (18 U) at 37°C for 2 hours, the restriction enzyme is then inactivated at 65°C for 15 minutes. The product is dialysed on a Millipore® VSWP013000 cellulose acetate and nitrate membrane against water for 2 hours. The double-stranded viral DNA AatII/AatII restriction

fragment thus prepared is frozen at -20°C while awaiting the ligation step.

The vector pcDNA2.1 is modified in order to receive the AatII fragment by site-directed insertion mutation: the EagI restriction site of the multiple cloning site was removed and replaced with an AatII site. The vector pcDNA2.1a thus produced was amplified in bacterial culture and purified with the aid of a QIAfilter Plasmid Maxi Kit (Qiagen S.A., France). Next, 3 µg of the vector pcDNA2.1a is subjected to restriction with the enzyme AatII at 37°C for 1 hour and then dephosphorylated with shrimp alkaline phosphatase (Boehringer Mannheim, Meylan, France). The enzymes are inactivated at 65°C for 15 minutes.

The ligation is carried out with a vector/viral DNA insert molar ratio of 1/1, that is to say 50 ng of vector and 100 ng of viral DNA insert, prepared as described above, with the aid of 1 U of T4 ligase (Life Technologies, France) at 24°C for 16 hours. After a 1/2 dilution, the ligation product is heated at 65°C in order to inactivate the T4 ligase and then cooled on ice. Electrocompetent bacteria Sure® (Stratagene, Heidelberg, Germany) are electrophorized with 2 or 4 µl of this ligation solution (1500 V, 50 µF, 200 Ω) and then incubated with 1 ml of SOC medium (Life Technologies, France) for 1 hour before being spread on a Luria Broth agar medium (Life Technologies, France) containing 100 µg/ml of amoxycillin, 15 µg/ml of tetracycline, 100 µg/ml of IPTG and 50 µg/ml of X-gal.

Twenty four (recombinant) white colonies were selected, their plasmid is extracted by minipreparation of DNA and a rough restriction map (AatII, AatII + BamHI, BamHI, BamHI + BglII, HindII) made it possible to select 2 recombinant clones with an insert having a size and a restriction map compatible with a V9 viral DNA insert.

These 2 clones (2 and 22) were sequenced with the aid of an automated sequencer ABI 377 (Perkin Elmer, France): they indeed contain an insert of

5028 bp, the 2 sequences are identified except at position 1165 (A and G for the clones 2 and 22 respectively). The direct sequence of the V9 viral DNA made it possible to determine that it is the G at position 1165 which is correct; it is therefore clone 22 which was selected (PCD.V9.C22), whose sequence corresponds to SEQ ID NO:1.

Figures 1 to 6 show the genetic distances which exist between erythrovirus V9 and erythrovirus B19. In these figures, the different erythrovirus sequences are represented by their mnemonic in GenBank (*release* 103.0 of October 1997).

EXAMPLE 2: Diagnosis of an erythrovirus type V9 by DNA hybridization (dot blot or slot blot or microplate) with a specific probe

The viral DNA is extracted, for example, with the aid of a QIAamp Blood Kit column (Qiagen S.A., France) or of any other method of extracting nucleic acids from a biological sample (blood, serum, plasma, amniotic fluid, bone marrow, tissue). The DNA in solution is denatured at 95°C for 2 minutes and then cooled on ice, transferred onto nylon or cellulose nitrate membrane by vacuum filtration and then fixed (heating of the membrane at 80°C for 1 hour). The membrane is then hybridized under stringent conditions with a DNA or RNA probe specific for V9, such as the sequence SEQ ID NO:1 or its complementary sequence or a fragment thereof, in particular the sequences SEQ ID NO:45 to SEQ ID NO:80 and 110 and their complementary sequences, or a fragment of these sequences which are appropriately labelled. This labelling may be a labelling with a radioelement (^{32}P, ^{33}P, ^{35}S, ^{3}H, ^{14}C or another radio isotope), a cold labelling (biotin), fluorescent marker, digoxygenin or any other molecule which may be coupled or incorporated into a DNA or RNA fragment and which can be detected by a specific antibody, or by a ruthenium chelate). In the case of a labelling with a radioactive element, the visualization is performed by autoradiography or any other method

allowing the detection of the radioisotope emission (such as Phosphorimager, Molecular Dynamics, Bondoufle, France). In the case of a labelling with biotin, the visualization is performed with the aid of an enzyme/streptavidine conjugate and a suitable visualization substrate. In the case of a fluorescent labelling, the visualization is made with the aid of a fluoro-Imager (Molecular Dynamics, Bondoufle, France) or any other apparatus capable of detecting the fluorescence emission. In the case of a labelling with digoxygenin (or with another antigen), the visualization is made with the aid of an anti-digoxgenin antibody (or an antibody specific for the antigen used for the labelling), coupled directly to an enzyme (alkaline phosphatase, peroxidase or any other enzyme), or in an indirect manner with an anti-digoxygenin antibody (or an antibody specific for the antigen used for the labelling) and an antibody coupled to an enzyme. A substrate suitable for the enzyme of the conjugate is used for the visualization. In the case of a labelling with ruthenium chelate (such as TBR), the visualization is performed by an electro-chemiluminescence reaction (G.F. Blackburn et al., Clin. Chem., 1991, 37:1534-1539).

A variant of this technique comprises the fixing of viral DNA on a microplate or another solid support and hybridization with a labelled probe as specified above.

Another variant of this technique comprises the fixing of an unlabelled probe on a microplate or another solid support and hybridization with the viral DNA of the sample which would have been labelled beforehand.

<u>EXAMPLE 3</u>: Diagnosis of an erythrovirus type V9 by gene amplification (PCR or *polymerase chain reaction*) and hybridization

Viral DNA is extracted from a biological sample (blood, serum, plasma, amniotic fluid, bone marrow, tissue) with the aid of a QIAamp Blood Kit column

(Qiagen S.A., France) or of any other method of extracting nucleic acids.

The PCR is carried out according to the method described by Saiki et al. (Nature, 1986, 324: 163-66) with 10 µl of DNA solution in a final volume of 100 µl of reaction mixture (50 mM KCl; 10 mM Tris-HCl pH 8.3; 2.5 mM $MgCl_2$; 200 µM dNTP; 25 pmol of sense and antisense oligonucleotides) with 1.5 IU of AmpliTaq Gold™ (Perkin Elmer, France). The amplification primers are oligonucleotides of 20 to 25 mers chosen so as to amplify only the DNA of the V9 variant: either the 2 primers (sense and antisense) are fragments of the sequences specific for V9 (SEQ ID NO:45 to 80, 108 and 110) or their complementary sequences, or one of the primers is chosen from the sequences specific for V9 (SEQ ID NO:45-80, 108 and 110) or their complementary sequences whereas the other primer is chosen from the sequences capable of hybridizing both the B19 erythroviruses and the V9 erythroviruses (SEQ ID NO:2 to 44, 105-107, 109 and 111-112) or their complementary sequences. The temperature cycles are applied to the reaction mixture by a thermocycler (T9600, Perkin Elmer, France) according to the following programme:

1 cycle:
- 6 minutes at 95°C

5 cycles:
- 60 seconds at 95°C
- 30 seconds at 60°C
- 30 seconds at 72°C

45 cycles:
- 30 seconds at 95°C
- 30 seconds at 60°C
- 30 seconds at 72°C

1 cycle:
- 5 minutes at 72°C

The product of amplification is deposited on a 1.3% agarose gel so as to be subjected to an electrophoretic separation and a transfer onto a nylon membrane loaded by capillarity according to a

conventional technique (Sambrook J. et al., 1989, *Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor*).

The probe is an oligonucleotide of 20-30 mers, a fragment of a sequence specific for V9 (SEQ ID NO:45 to 80, 108 and 110) or their complementary sequences. It is labelled in 3' with DIG-dUTP with the aid of the DIG *Oligonucleotide Tailing* kit (Boehringer Mannheim, Meylan, France).

The transfer membrane is prehybridized in a buffer comprising (50% formamide; 5X SSC; 2% of blocking reagent (Boehringer Mannheim, Meylan, France); 0.1% of N-laurylsarcosine; 0.02% of SDS), at 42°C for 90 minutes. The hybridization is carried out in 3 ml of a buffer of the same composition with 10 µl of labelled probe at 42°C for 16 hours. The membrane is washed twice in 2X SSC buffer containing 0.1% SDS at 60°C for 10 minutes, and then twice in 1X SSC buffer containing 0.1% SDS at 60°C for 10 minutes. The membrane is then visualized with DIG *Luminescent Detection Kit* (Boehringer Mannheim, Meylan, France) and an autoradiography.

EXAMPLE 4: Group diagnosis and differential diagnosis of type B19 and V9 erythroviruses by gene amplification and hybridization

The viral DNA is extracted from a biological sample (blood, serum, plasma, amniotic fluid, bone marrow, tissue) with the aid of a QIAamp Blood Kit column (Qiagen S.A., France) or of any other method of extracting nucleic acids.

The PCR is carried out according to the method described by Saiki et al. (Nature, 1986, cited above) with 10 µl of DNA solution in a final volume of 100 µl of reaction mixture (50 mM KCl; 10 mM Tris-HCl pH 8.3; 2.5 mM $MgCl_2$; 200 µM dNTP; 25 pmol of sense and antisense oligonucleotides) with 1.5 IU of AmpliTaq Gold™ (Perkin Elmer, France). The amplification primers are oligonucleotides of 20 to 25 mers chosen so as to amplify the DNA of B19 and of the variant V9: the 2

(sense and antisense) primers are fragments of the sequences capable of hybridizing both with the B19 erythroviruses and with the V9 erythroviruses (SEQ ID NO:2 to 44, 105-107, 109, 111-112) or of their complementary sequences. The temperature cycles are applied to the reaction mixture by a thermocycler (T9600, Perkin Elmer, France) according to the following programme:

1 cycle:
- 6 minutes at 95°C

5 cycles:
- 60 seconds at 95°C
- 30 seconds at 60°C
- 30 seconds at 72°C

45 cycles:
- 30 seconds at 95°C
- 30 seconds at 60°C
- 30 seconds at 72°C

1 cycle:
- 5 minutes at 72°C

The product of amplification is deposited on a 1.3% agarose gel so as to be subjected to an electrophoretic separation and a transfer onto a nylon membrane loaded by capillarity according to a conventional technique (Sambrook J. et al., 1989, cited above).

The probe is an oligonucleotide of 20-30 mers, a fragment of a sequence specific for V9 (SEQ ID NO:45 to 80, 108 and 110) or their complementary sequences, or alternatively specific for B19, or finally which hybridizes both with B19 and with V9 (SEQ ID NO:2 to 44 or 105-107, 109, 111-112), if it is sought to carry out a group diagnosis. It is labelled in 3' with DIG-dUTP with the aid of the DIG *Oligonucleotide Tailing* kit (Boehringer Mannheim, Meylan, France).

The transfer membrane is prehybridized and hybridized under the same conditions as those set out in Example 3.

EXAMPLE 5: Group diagnosis and differential diagnosis of type B19 and V9 erythroviruses by gene amplification and restriction enzymes

Extraction of the viral DNA from a biological sample (blood, serum, plasma, amniotic fluid, bone marrow, tissue) with the aid of a QIAamp Blood Kit column (Qiagen S.A., France) or of any other method of extracting nucleic acids.

The NS1a PCR is carried out according to the method described by Saiki et al. with 5 µl of DNA solution in a final volume of 50 µl of reaction mixture (50 mM KCl; 10 mM Tris-HCl pH 8.3; 2.5 mM $MgCl_2$; 200 µM dNTP; 12.5 pmol of sense and antisense oligonucleotides) with 1.5 IU of AmpliTaq Gold™ (Perkin Elmer, France) and the pair of primer B (sense primer e1905f, SEQ ID NO:105; and the antisense primer e1987r, SEQ ID NO:106) using the following temperature cycles (on a thermocycler T9700, Perkin Elmer, France):

1 cycle:
- 6 minutes at 94°C

5 cycles:
- 30 seconds at 94°C
- 1 minute at 55°C
- 1 minute at 72°C

45 cycles:
- 30 seconds at 94°C
- 30 seconds at 60°C
- 30 seconds at 72°C

1 cycle:
- 7 minutes at 72°C

An aliquot of the product of amplification (10 µl) was deposited on a 2% agarose gel so as to be subjected to an electrophoretic separation and a transfer onto a nylon membrane loaded by capillarity according to a conventional technique (J. Sambrook et al., 1989, cited above). The membrane was hybridized with an oligonucleotide probe of 36 mer, e1954fp (SEQ ID NO:121): ACCAGTATCAGCAGCAGTGGTGGTGAAAGCTCTGAA, a

fragment of the sequence SEQ ID NO:11. This probe allows a detection of type B19 and V9 erythroviruses.

An aliquot of the product of amplification (10 µl) was subjected to the action of the restriction enzyme MunI for 2 hours and then subjected to an electrophoretic separation on a 2% agarose gel. As described above, the erythrovirus type is B19 if there is cleavage, and V9 if there is no cleavage.

Results of the NS1a PCR:

79 samples found to be indeterminate or weakly positive with the old B19 PCR (Lefrere, et al., Transfusion, 1995, 35:389-391) were screened with the aid of the new NS1a PCR (consensus erythrovirus, sequences according to the invention). Of the 79 samples screened, 31 are positive and were typed with the aid of the restriction enzyme MunI: 18 (58%) were found to be of type B19 and 13 (42%) of type V9.

The samples which were positive by NS1a PCR were able to be amplified on 1100 bp by a nested PCR (S1S2 PCR) with the aid of the pair of primers e1855f (SEQ ID NO:113) and e2960r (SEQ ID NO:114) for the first amplification step of 30 cycles (PCRS1), and of the pair of primers e1863f (SEQ ID NO:115) and e2953r (SEQ ID NO:116) for the second amplification step of 50 cycles (PCRS2). 15 samples were found to be positive by S1S2 PCR and sequenced on 1110 bp (13 of type B19 by NS1A PCR and 2 of the variant type). The analysis of the sequences showed that:

- the B primers (sense primer e1905f, SEQ ID NO:105; and antisense primer e1987r, SEQ ID NO:106), are perfectly conserved for all the 15 sequences (of the B19 and variant type) as well as for all the known B19 sequences, confirming their importance for use for a consensus diagnostic test for B19 and V9,

- the probe e1954fp (SEQ ID NO:121), a fragment of the sequence SEQ ID NO:11 is equally well conserved for the 15 sequences as well as for all the known B19 sequences,

- the B19 sequences form a well homogeneous group with less than 1.2% divergence between them (7 B19 sequences of GenBank and the 13 B19 sequences of this study),

- finally for the 2 sequences typed variant erythrovirus by NS1a PCR with MunI digestion, less than 4.5% divergence with V9 is observed.

EXAMPLE 6: Cloning of the capsid genes VP1 and VP2 of V9 into a baculovirus expression vector

First step:

- cloning of the VP1 gene into a bacterial plasmid

The VP1 gene of V9 is amplified by PCR according to the method described by Saiki et al. (Nature, 1986, 324:163-166) with 10 µl of a 10^{-2} dilution of V9 viral DNA in a final volume of 100 µl of reaction mixture (20 mM Tris-HCl pH 8.8; 10 mM KCl, 10 mM $(NH_4)_2SO_4$; 2 mM $MgSO_4$; 0.1% Triton X-100; 0.1 mg/ml of BSA; 0.2 mM dNTP; 25 pmol of sense primers (e2435fStuI/BglII: AAAGGCCTAGATCTTGTAGATTATGAGTAAAAC, SEQ ID NO:117) and antisense primers (e4813rEcoRI: GGGAATTCGGTGGGTGACGGTTCCTG, SEQ ID NO:118) with 2.5 U of Pfu Turbo™ (Stratagene, France). The amplification primers were chosen on the V9 sequence on either side of the VP1 gene, their 5' end was modified by addition of restriction site(s) (indicated in their name) in order to facilitate the cloning. The temperature cycles applied to the reaction mixture are the following:

1 cycle:

- 1 minute at 94°C

20 cycles:

- 1 minute at 94°C
- 1 minute at 55°C
- 2.5 minutes at 72°C

1 cycle:

- 10 minutes at 72°C

The product of amplification of the VP1 gene was purified with the aid of a silica column (QIAquick PCR Purification Kit, Qiagen, France) and then

subjected to the action of the restriction enzymes StuI and EcoRI. After heat inactivation of the restriction enzymes (20 min at 65°C), the VP1 gene fragment was purified by dialysis against H_2O on a 0.025 µm filter (VSWP01300, Millipore).

The plasmid pBacPAK8 (Clontech, France) is subjected to the action of the restriction enzymes StuI and EcoR1, the vector is then dephosphorylated with shrimp alkaline phosphatase (Boehringer, France). After heat inactivation of the restriction enzymes (20 min at 65°C), the plasmid was purified with the QIAquick PCR Purification Kit (Qiagen).

The ligation is carried out with 50 ng of plasmid pBacPAK8 and 100 ng of VP1 fragment (prepared as described above) with T4 ligase (Life Technologies, France). After heat inactivation of the T4 ligase (10 min at 65°C), 2 µl of ligation product diluted 1/2 with water are electroporated with 25 µl of electro-competent bacteria (Epicurian Coli Sure Electroporation-Competent cells, Strategene). The electroporated bacteria are immediately taken up in 1 ml of SOC medium (2% tryptone, 0.5% of yeast extracts, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose), incubated for 1 h at 37°C, with stirring. Next, 10 µl, 100 µl and 890 µl of the transformed bacteria are plated on Lennox agar dishes (10 g/l of peptone, 5 g/l of yeast extracts, 5 g/l NaCl, and 13 g/l agar) containing 50 µg/ml of ampicillin. After 24 h of incubation at 37°C, 24 colonies per construct are subcultured in 5 ml of Lennox medium with 50 µg/ml of ampicillin and incubated for 24 h at 37°C, with stirring.

The plasmid DNA is extracted by alkaline minilysis with the aid of the QIAprep 8 Turbo miniprep kit (Qiagen) and analysed by StuI/EcoRI and KpnI/-HindIII restriction in order to determine the presence of the insert and its orientation. The clone pB8-VP1.C5 was selected and the recombinant plasmid was checked by sequencing.

- cloning of the VP2 gene into a bacterial plasmid.

The VP2 gene of V9 is amplified by PCR according to the method described by Saiki et al. (Nature, 1986, 324:163-166) with 10 µl of a 10^{-2} dilution of V9 viral DNA in a final volume of 100 µl of reaction mixture (20 mM Tris-HCl pH 8.8; 10 mM KCl, 10 mM $(NH_4)_2SO_4$; 2 mM $MgSO_4$; 0.1% Triton X-100; 0.1 mg/ml of BSA; 0.2 mM dNTP; 25 pmol of sense primers (e3115fBamHI: CACGGATCCATACCCCAGCATGACTTCAG, SEQ ID NO:119) and antisense primers (e4813rBamHI: CACGGATCCGGTGGGTGACGGTTCCTG, SEQ ID NO:120) with 2.5 U of Pfu Turbo™ (Stratagene, France). The amplification primers were chosen on the V9 sequence on either side of the VP2 gene, their 5' end was modified by addition of restriction site(s) (indicated in their name) in order to facilitate the cloning. The temperature cycles applied to the reaction mixture are the following:

1 cycle:

- 1 minute at 94°C

20 cycles:

- 1 minute at 94°C
- 1 minute at 60°C
- 2.5 minutes at 72°C

1 cycle:

- 10 minutes at 72°C

The product of amplification of the VP2 gene was purified with the aid of a silica column (QIAquick PCR Purification Kit, Qiagen, France) and then subjected to the action of the restriction enzymes BamHI. The VP2 gene fragment was purified by the QIAquick PCR Purification Kit (Qiagen).

The plasmid pBacPAK8 (Clontech, France) is subjected to the action of the restriction enzymes BamHI, the vector is then dephosphorylated with shrimp alkaline phosphatase (Boehringer, France). After heat inactivation of the shrimp alkaline phosphatase (20 min at 65°C), the plasmid was purified by phenol/chloroform extraction and precipitated with ethanol.

The ligation is carried out with 50 ng of plasmid pBacPAK8 and 100 ng of VP2 fragment (prepared as described above) with T4 ligase (Life Technologies, France). After heat inactivation of the T4 ligase (10 min at 65°C), 2 µl of ligation product diluted 1/2 with water are electroporated with 25 µl of electro-competent bacteria (Epicurian Coli Sure Electro-poration-Competent cells, Stratagene). The electroporated bacteria are immediately taken up in 1 ml of SOC medium, incubated for 1 h at 37°C, with stirring. Next, 10 µl, 100 µl and 890 µl of the transformed bacteria are plated on Lennox agar dishes containing 50 µg/ml of ampicillin. After 24 h of incubation at 37°C, 24 colonies per construct are sub-cultured in 5 ml of Lennox medium with 50 µg/ml of ampicillin and incubated for 24 h at 37°C, with stirring.

The plasmid DNA is extracted by alkaline minilysis with the aid of the QIAprep 8 Turbo miniprep kit (Qiagen) and analysed by BamHI and SacI restriction in order to determine the presence of the insert and its orientation. The clone pB8-VP2.C20 was selected and the recombinant plasmid was checked by sequencing: a base A deleted just upstream of the initiator ATG of VP2 can be noted, but this mutation can be ignored: it will not generate the expression of VP2.

Second step:

- Construction of the recombinant baculovirus expressing VP1

The plasmid pB8-VP1.C5 is cotransfected with the baculovirus BacPAk6, linearized with Bsu361 (BacPAK™ Baculovirus Expression System, Clontech), into SF9 insect cells with lipofectin. 2 isolations are performed by the lysis plaque method, the plaques isolated are transferred onto a nitrocellulose membrane, the membrane is then hybridized with a DNA probe specific for the VP1 gene of V9.

The recombinant baculovirus BacPAK6-pB8-VP1.C4.2 was thus selected. The expression of the VP1

protein was verified by Western Blotting on a cellular pellet of SF9 cells infected with this recombinant baculovirus. A band was observed at the expected size of VP1 (about 80 kDa) but which is not recognized by the anti-VP1-B19 monoclonal antibody (Argène, France). It is possible that this monoclonal antibody does not crossreact with the VP1 protein of V9.

The cloning into a baculovirus was verified by sequencing after PCR with the primers Bac1 and Bac2 (Clontech).

- Construction of the recombinant baculovirus expressing VP2

The plasmid pB8-VP2.C20 is cotransfected with the baculovirus BacPAk6, linearized with Bsu361 (BacPAK™ Baculovirus Expression System, Clontech), into SF9 insect cells with lipofectin. 2 isolations are performed by the lysis plaque method, the plaques isolated are transferred onto a nitrocellulose membrane, the membrane is then hybridized with a DNA probe specific for the VP2 gene of V9.

The recombinant baculovirus BacPAK6-pB8-VP2.-C1.3 is selected. The expression of the VP2 protein was verified by Western Blotting on a cellular pellet of SF9 cells infected with this recombinant baculovirus. The anti-VP2-B19 monoclonal antibody (Argène, France) indeed detects a protein with an apparent molecular weight of about 58 kDa which is also clearly visible on the acrylamide gel. Virus-like particles of about 20 to 30 nm in diameter are observed by electron microscopy in the culture supernatants of the SF9 cells after infection with a recombinant baculovirus expressing the VP2 protein of V9. The size and the appearance of the virus-like particles obtained are in every respect in conformity with those described for B19. This observation confirms that the VP2 protein of V9 is produced in a native form by the baculovirus, because it is capable of forming empty capsides by self-assembling.

The cloning into a baculovirus was verified by sequencing after PCR with the primers Bac1 and Bac2 (Clontech).

Third step:

The proteins VP1 and VP2 of V9 expressed in a baculovirus will be purified so as to be used as a target antigen for new serological tests for the diagnosis of erythrovirus V9 infections.

As is evident from the above, the invention is not at all limited to its embodiments, implementations and applications which have just been described more explicitly; it encompasses on the contrary all the variants which may occur to the specialist in this field, without departing from the framework or the scope of the present invention.